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Trypsinogen Expression and Early Detection for Peritoneal Dissemination in Gastric Cancer

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Background and Objectives: The most reliable method for the diagnosis of peritoneal dissemination of gastric cancer at the present time is cytological examination of ascitic fluid, which is unavailable to patients without ascites or may be inadequate for those with ascites containing few cancer cells. It has been reported recently that human gastric cancer immunoreacted with a monoclonal antibody against pancreatic trypsinogen. We therefore examined the expression of trypsinogen as a new marker for the early diagnosis of peritoneal dissemination of gastric cancer.

Methods: Pancreatic trypsinogen protein was immunohistochemically stained with a three-step indirect immunoperoxidase method and cationic trypsinogen (trypsinogen-1) mRNA expression was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in gastric cancer. Twenty-nine of 30 primary tumors (97%) and all 12 tumors (100%) of the peritoneal seedings immunohistochemically reacted with trypsinogen. Preliminary study for early diagnosis of peritoneal dissemination was carried out for eight more recent patients who showed positive immunoreactivity to trypsinogen protein and expressed trypsinogen-1 mRNA in the primary tumor. The expression of trypsinogen-1 mRNA was detected by using peritoneal lavage fluid preoperatively collected in these patients.

Results: All three patients in whom peritoneal dissemination was diagnosed at the time of their operation(s) expressed trypsinogen-1 mRNA. One patient, who did not show peritoneal dissemination at the operation but was positive for trypsinogen-1 mRNA detection, later died of the recurrence of peritoneal dissemination.

Conclusions: These results indicated that trypsinogen protein and trypsinogen-1 mRNA frequently expressed in peritoneal dissemination as well as primary tumors in gastric cancer and detection of trypsinogen-1 mRNA expression was a useful method for early diagnosis in peritoneal dissemination of gastric cancer. *J. Surg. Oncol. 1998;69:71–75.* © 1998 Wiley-Liss, Inc.

KEY WORDS: peritoneal dissemination; gastric cancer; pancreatic trypsinogen-1; RT-PCR

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INTRODUCTION

It is important to make an exact and early diagnosis of peritoneal dissemination since the prognosis of far advanced peritoneal dissemination in gastric cancer is extremely poor and there is no effective treatment for this disease. Laparoscopic examination is reported useful for the diagnosis of peritoneal dissemination in spite of its invasiveness [1]. However, it never discloses peritoneal dissemination in a latent phase because assessment by laparoscopy depends upon macroscopic findings. The diagnosis of this disease is accordingly difficult until patients suffer from massive ascites, bulky abdominal tumor, or intestinal obstruction. At present, cytological examination of ascites is the most reliable method for the detection of peritoneal dissemination. Cytological examination, however, is unavailable for patients without ascites and may be inadequate for those with ascites containing few cancer cells.

We previously reported the usefulness in the early diagnosis of peritoneal dissemination by epithelial-cadherin (E-cadherin) mRNA expression in cells, which were preoperatively collected from ascites or intraperitoneally infused fluid, with the reverse transcriptase-polymerase chain reaction (RT-PCR) technique [2]. However, the sensitivity of this method was not very high, especially for far advanced peritoneal dissemination. It was suspected to be because of reduced expression of E-cadherin in poorly differentiated adenocarcinoma, which frequently developed peritoneal dissemination. Recently, it was reported that human gastric cancer immunoreacted with a monoclonal antibody against pancreatic trypsinogen [3]. In this study, we examined the expression of trypsinogen as a new marker for the early diagnosis of peritoneal dissemination of gastric cancer.

MATERIALS AND METHODS

Immunochemistry

Thirty specimens of primary gastric cancers and 12 tumors of peritoneal seedings of gastric cancers were examined for the immunohistochemical study. These 30 patients were 19 men and 11 women, and their ages ranged between 42 and 78 years with a mean age of 58 years. All of the primary gastric cancers examined in this series were histologically proven to be poorly differentiated adenocarcinoma. Pancreatic trypsinogen immunohistochemistry was performed with a three-step indirect immunoperoxidase method (streptavidin-biotin-peroxidase complex). Briefly, sections were deparaffinized with graded xylene and alcohol solutions. Then, protease digestion was applied using protease K (Boehringer Mannheim Biochemica, Mannheim, Germany) at a concentration of 40 μg/ml for 10 min at room temperature to facilitate penetration of the primary antibody [4]. Following a phosphate-buffered saline (PBS) rinse, the sections were immersed in absolute methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity. Following a PBS rinse, the sections were incubated with normal goat serum at a 1:30 dilution for 30 min at room temperature to block non-specific binding. Monoclonal antibody against human pancreatic trypsinogen (dilution = 1:100; Chemicon International, Inc., Temecula, CA) was added and incubated at 4°C overnight. The sections were then treated with biotinylated goat anti-mouse IgG (Dakopatts, Copenhagen, Denmark) for 30 min. Peroxidase-labeled streptavidin (Dakopatts) was then added to sections for 30 min at room temperature. Reaction products were developed by immersing the sections in 3.3′-diaminobenzidine tetrahydrochloride solution containing 0.1% H_2O_2. Slides were counterstained lightly with methyl green. In each immunostaining run, the primary antibodies were replaced by non-immune normal mouse serum (Dako, Santa Barbara, CA) as negative controls. Sections from normal human pancreas were used as positive controls.

RT-PCR

In eight more recent patients, not only immunoreactivity to trypsinogen protein, but also expression of cationic trypsinogen (pancreatic trypsinogen-1) [5] mRNA of the primary tumors and expression of trypsinogen-1 mRNA of the peritoneal lavage fluid preoperatively collected were investigated as a preliminary study for early diagnosis of peritoneal dissemination. The detection of trypsinogen-1 mRNA was carried out by RT-PCR, for which the samples were immediately frozen in liquid nitrogen and stored at −8°C. Total RNA was extracted from these stored samples by using the acid guanidium-phenol-chloroform technique with ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of total RNA and 50 pmol of oligo dT were mixed, denatured at 68°C for 15 min, and then immediately chilled on ice for 10 min. In the reaction buffer [50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 0.5 mM each dNTPs, 225 μg/ml of bovine serum albumin, 5 mM of dithiothreitol, 20 units of RNasin, and 4 units of AMV reverse transcriptase (Life Science, St. Petersburg, FL)], the samples were incubated at 40°C for 90 min, at 95°C for 5 min, and then snap cooled. Then all samples of cDNA were amplified on a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) by the PCR method. Samples of cDNA were amplified by 30 cycles of PCR with the primer pair specific to the pancreatic trypsinogen-1, 5′-ACCACCATGAATCCACTCCTG-3′ and 5′-GCTTTAGCTATTGGCAGCTAT-3′. As an internal standard, primer pairs specific to the β-actin gene, 5′-GAAAATCTGGCACCACACCTT-3′ and 5′-GTTGAAGGTAGTTTCGTGGAT-3′, were used. The reaction mixture contained 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 0.5 mM each dNTPs, 50 pmol

of each sense and antisense primers, and 2.5 units of Taq polymerase (Takara Shuzo, Kyoto, Japan). The amplification was performed with denaturation at 94°C for 30 sec, annealing at 62°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed on a 2.0% agarose gel containing ethidium bromide and subsequently transferred to a nylon membrane. Hybridization was carried out with the ^{32}P-end-labeled probe specific for the target cDNA fragment [6]. The autoradiogram of the membrane was analyzed by a BAS 2000 FUJIX imaging analyzer (FIJIX, Tokyo, Japan).

Clinical Preparation

The specimen of intraperitoneal lavage fluid was preoperatively obtained by abdominal paracentesis using an 8 Fr trocar catheter under local anesthesia. About 1,000 ml saline was infused into the peritoneal cavity and then collected after the patient was rolled from side to side. The sample was divided into two parts, with one subjected to cytological examination and the other used for the RT-PCR analysis of trypsinogen-1 mRNA.

The final diagnosis of peritoneal dissemination was made at operation or autopsy based on the macroscopic and microscopic findings. The grading of peritoneal dissemination was based on ''The General Rules for the Gastric Cancer Study (12th Ed.)'' [7]. No dissemination was graded as P_0; slight as P_1; moderate as P_2; and severe dissemination as P_3. By cytological examination the cells were categorized as class I, normal; class II, a few atypical cells; class III, dysplasia; class IV, carcinoma in situ; and class V, overt cancer [8]. In this study, class IV and class V were considered to be positive for malignancy.

RESULTS

Twenty-nine of 30 primary tumors (97%) immunohistochemically reacted with pancreatic trypsinogen (Fig. 1). The immunoreactive pattern was coarsely granular and was generally present in the supranuclear cytoplasm of the cancer cells. The intensity of pancreatic trypsinogen immunoreactivity in the tumor area varied from specimen to specimen, as well as from area to area within the same specimen. In most cases, trypsinogen immunoreactivity was more pronounced at advanced edges of the tumor. All 12 tumors (100%) of the peritoneal seedings were immunohistochemically positive for pancreatic trypsinogen (Fig. 2). Immunohistochemical staining showed clearly.

RT-PCR analysis for trypsinogen-1 mRNA in both primary tumors and peritoneal lavage fluids was examined for eight recent primary tumors of patients who showed positive immunoreactivity to trypsinogen protein. Expression of trypsinogen-1 mRNA of primary tumor was confirmed in all eight patients (Fig. 3). The pancreas tissue and the liver tissue are a positive control and a negative control, respectively.

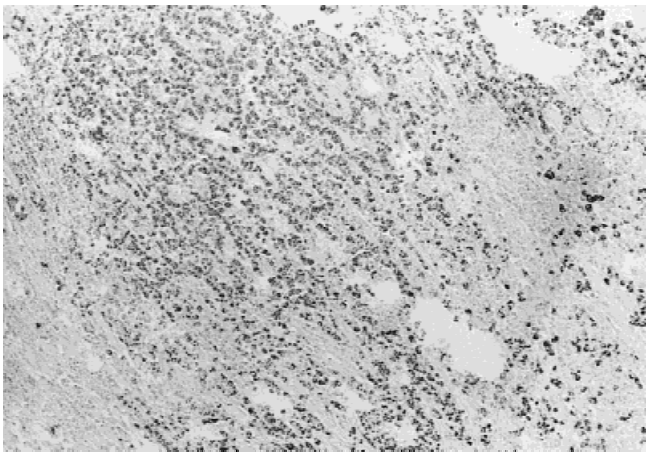

Fig. 1. Immunohistochemistry of pancreatic trypsinogen for the primary lesion of gastric cancer. The immunoreactive pattern of trypsinogen was coarsely granular and was generally present in the supranuclear cytoplasm of the cancer cells. Twenty-nine of 30 primary tumors (97%) immunohistochemically reacted with trypsinogen.

Fig. 2. Immunohistochemistry of pancreatic trypsinogen for peritoneal disseminations of gastric cancer. The cancer cells infiltrating with gland formation were stained with trypsinogen. All four tumors of the peritoneal seedings were immunohistochemically positive for trypsinogen.

These eight patients were preoperatively examined for trypsinogen-1 mRNA detection with the PCR method by using peritoneal lavage fluid. Three of eight patients who were examined for trypsinogen-1 mRNA by peritoneal lavage fluid showed peritoneal dissemination at operation (Fig. 4). The degree of peritoneal dissemination was P_1 in case 3, P_2 in case 5, and P_3 in case 1. All of these three patients were positive for trypsinogen-1 mRNA, but one patient (case 3) had a negative cytology. The other five patients did not have peritoneal dissemination at their operation, but three patients (cases 4, 6, and 7) were positive for trypsinogen-1 mRNA. One patient died of recurrent peritoneal dissemination (case 4). It was necessary for two other patients (case 6 and 7) to be followed up carefully, because the detection of trypsinogen-1

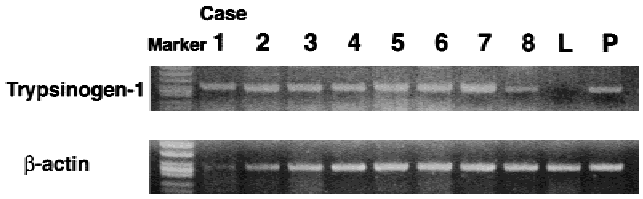

Fig. 3. RT-PCR analysis for trypsinogen-1 mRNA in the patients who showed positive immunoreactivity to trypsinogen protein. Expression of trypsinogen-1 mRNA was confirmed in all eight patients examined. The pancreas tissue and the liver tissue were positive and negative controls, respectively.

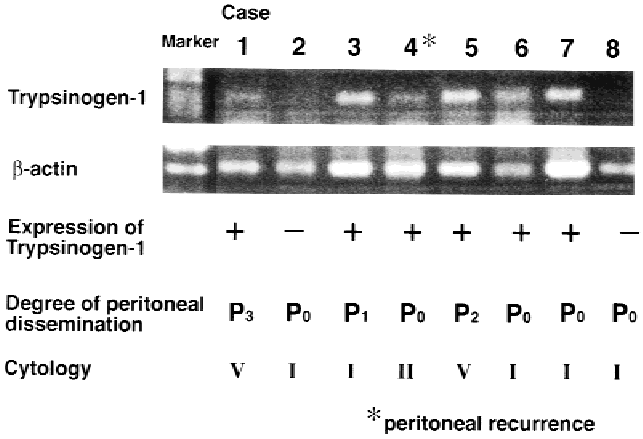

Fig. 4. RT-PCR analysis for trypsinogen-1 mRNA by using peritoneal lavage fluid preoperatively collected in eight patients. All of three patients who showed peritoneal dissemination at their operation (cases 1, 3, and 5) were positive for trypsinogen-1 mRNA, but one patient (case 3) had a negative cytology. One patient (case 4), who did not show peritoneal dissemination at the operation but was positive for trypsinogen-1 mRNA, died of recurrent peritoneal dissemination.

mRNA might indicate the presence of occult cancer cells that were not detected by either macroscopic or cytological examination.

DISCUSSION

Infiltrative gastric cancer frequently develops peritoneal dissemination. The formation of the dissemination needs defoliation of cancer cells from the invaded serosal surface, attachment to mesothelial cells or extracellular matrix under the mesothelium, and invasive growth in extracellular matrix. While these sequential phenomena are suspected to involve many genetic alternations, various proteinases such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activators (uPAs) play an important role in the invasion by cancer cells into the extracellular matrix. MMP-2, MMP-9, and MT-MMP were reported to be strongly related with the invasion of cancer cells [9,10]. Suzuki et al. [9] indicated that production and activation of proMMP-2 correlate directly with tumor cell invasion and lymphatic permeation in gastric cancer. Our previous immunohistochemical study using a monoclonal antibody to uPA showed that in gastric cancer the uPA-positive tumors had a higher frequency of peritoneal dissemination than the uPA-negative ones [11].

Pancreatic trypsinogen, a serine protease, is one of the digestive enzymes produced by pancreatic acinar cells. The expression of trypsinogen protein was reported in human pancreatic adenocarcinoma cell lines CFPAC-1 and CAPAN-1 [12], as well as in invasive ductal adenocarcinoma tissues of the pancreas [13]. Koshikawa et al. [14] showed that two of ten human gastric cancer cell lines, STKM-1 and MKN-28, secreted trypsinogen by means of immunoblotting as well as gelatin zymography, and identified one- and two-chain forms of pancreatic trypsinogen-1 in a serum-free conditioned medium of the STKM-1 cell line [15]. Moreover, it was demonstrated that human gastric carcinomas frequently immunoreacted with a monoclonal antibody against human pancreatic trypsinogen [3]. In this study, the trypsinogen protein was immunohistochemically positive in peritoneal seedlings as well as in primary gastric cancer.

In this study, one of three patients, who did not show macroscopic or cytological peritoneal dissemination but was positive for trypsinogen-1 mRNA, died of recurrent peritoneal dissemination. This fact might mean that occult cancer cells not detected by either macroscopic or cytological examination at the operation developed peritoneal dissemination thereafter. It is important for the patients who were preoperatively found to be positive for trypsinogen-1 mRNA to be prophylactically treated against recurrence of peritoneal dissemination and to be elaborately observed for their survival. For the patients with gastric cancer invading the serosal surface we have performed continuous hyperthermic peritoneal perfusion for the prevention of peritoneal recurrence [16].

PCR analysis of specific gene expression has already been reported for the early detection of micrometastases in lymph nodes [17]. For early diagnosis of peritoneal dissemination we have introduced the RT-PCR technique by using mRNA extracted from cells in ascites or intraperitoneal lavage fluid. We selected primers of the E-cadherin, epithelium-specific adhesion molecule [18] for the diagnosis of peritoneal dissemination [2] because the presence of epithelial cells shown by the expression of E-cadherin mRNA in the specimen indicates the presence of cancer cells when there are usually no epithelial cells in the peritoneal cavity. However, the rate of detection in peritoneal dissemination was not always high in our results. It is a cause of low sensitivity of our study that E-cadherin expression is much reduced in poorly differentiated gastric cancer, which frequently develops peritoneal dissemination [19]. Since trypsinogen-1 mRNA was expressed even in poorly differentiated adenocarcinoma of the stomach, this molecule is a good

candidate for the early detection of peritoneal dissemination.

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